American Journal of Hematology 54:288–295 (1997)

Differences Between Platelet Phosphoinositide Metabolism Stimulated by Thrombin or SFLLRN Are Not Accounted for by Interaction of Thrombin With Glycoprotein Ib

John D. Vickers,[1]* Marian A. Packham,[2] and Raelene L. Kinlough-Rathbone[1]

[1]Department of Pathology, McMaster University, Hamilton, Ontario, Canada
[2]Department of Biochemistry, University of Toronto, Toronto, Ontario, Canada

The formation of inositol phosphates was compared in aspirin-treated, washed human platelets suspended in Tyrode's-albumin solution containing 2 mM calcium and stimulated with SFLLRN (thrombin receptor-activating peptide) or thrombin. SFLLRN (20 μM) and thrombin (1 U/ml) resulted in maximal irreversible aggregation and 80–90% secretion of dense granule contents. SFLLRN (50–100 μM) caused larger increases at 10 sec than 20 μM SFLLRN in the formation of inositol trisphosphate (IP_3, measured as [^{3}H]inositol label). These increases were not significantly less than those caused by thrombin (1 unit/ml). However, whereas the labeling of IP_3 increased from 10–60 sec with thrombin, with SFLLRN it was much less at 60 sec than that at 10 sec. The decrease was not due to degradation of SFLLRN by ectopeptidases, since it was not prevented by amastatin, an inhibitor of ectopeptidases. Degradation of glycoprotein Ib (GPIb) with an O-sialoglycoprotein endopeptidase did not affect the thrombin-stimulated labeling of inositol phosphates, indicating that binding to GPIb is not involved in the sustained thrombin-induced formation of inositol phosphates. The finding that the thrombin-stimulated formation of IP_3 was not dependent on Ca^{2+} in the medium (EGTA added) indicates that the transient SFLLRN-induced formation of IP_3 is not due to failure to cause Ca^{2+} influx. The finding that formation of IP_3 was transient in SFLLRN-stimulated platelets, whereas platelet aggregation and secretion were maximal, indicates that the sustained activation of phospholipase C caused by thrombin may have roles related to later processes in which platelets participate. Am. J. Hematol. 54:288–295, 1997.

Key words: blood platelets; inositol phosphates; thrombin receptors

INTRODUCTION

Studies with peptides duplicating the sequence of amino acids exposed by thrombin cleavage of the extracellular portion of the seven transmembrane domain thrombin receptor have established that these peptides can stimulate many of the responses induced by thrombin [1], including activation of phospholipid metabolism [2]. SFLLR is the shortest effective member of this group [3,4]; in platelets the six amino-acid peptide, SFLLRN, appears to have the highest activity, in comparison with longer and shorter sequences [3–5].

However, differences between the response of platelets to thrombin and the peptide SFLLRN have been identified. Kinlough-Rathbone et al. [6] showed that whereas 20 μM SFLLRN, like 1 U/ml of thrombin, causes the maximal secretion of serotonin, the extent of thromboxane A_2 (TXA_2) synthesis is much lower (about 10%). Nieuwland et al. [7], in examining the stimulation of Na^+/H^+ exchange, Ca^{2+} mobilization, and protein kinase activities in platelets with thrombin (approximately 0.5 U/ml) or SFLLRN (15 μM), found the effect of SFLLRN to be reversible, in contrast to the effect of

Contract grant sponsor: Heart and Stroke Foundation of Ontario; Contract grant number: B-2447; Contract grant sponsor: Medical Research Council of Canada; Contract Grant number: MT 1309.

*Correspondence to: Dr. J.D. Vickers, Department of Pathology, HSC 4N58, McMaster University, Hamilton, Ontario L8N 3Z5, Canada.

Received for publication 29 June 1996; Accepted 6 November 1996.

thrombin. Based on comparisons of the effects of α-thrombin, γ-thrombin, and trypsin, they proposed that the cleavage of the receptor to form the tethered ligand was required for the sustained responses elicited by the proteases. Kramer et al. [8] found a similar transient increase in internal Ca^{2+} in SFLLRN-stimulated human platelets, which they concluded was due to a failure of SFLLRN to cause Ca^{2+} influx subsequent to the internal mobilization of Ca^{2+}. In another report, Lau et al. [9] proposed that SFLLRN acted as a partial agonist of the thrombin receptor, based on dose-response assessments, which would explain the differences between the effects of thrombin and SFLLRN. It is also possible that either the tethering of ligand SFLLRN and/or the guidance by thrombin of the tethered ligand to its binding site may result in more efficient interaction of the SFLLRN sequence with the binding site, and may account for the differences in efficacy of the tethered and untethered SFLLRN sequence [10]. In addition to these observations of differences between the action of thrombin and of SFLLRN, more than one receptor for thrombin on platelets has been proposed, based on evidence for binding sites with different affinities and different effects [11]. The cloned, tethered-ligand receptor is likely the medium-affinity receptor [12]. A high-affinity receptor, which has been suggested to be a multimolecular complex involving glycoprotein GPIb, GPIX, and GPV [11], has also been suggested to be responsible for activation of arachidonate metabolism [12].

We examined the effects of SFLLRN on the formation of inositol phosphates and compared the effects to those of thrombin. In all studies, platelets were treated with aspirin to block TXA_2 formation, since SFLLRN stimulates TXA_2 formation weakly compared to thrombin [6]. To examine the possible role of the interaction of thrombin with GPIb in changes in inositol phospholipid metabolism, the effects of degradation of GPIb with an O-sialoglycoprotease [13] were examined.

MATERIALS AND METHODS

Materials

SFLLRN was synthesized by the Institute for Molecular Biology and Biotechnology (MOBIX) at McMaster University, Hamilton, Ontario, dissolved at a concentration of 1 mM in isotonic saline and stored in aliquots at −70°C. [^{51}Cr]Na_2CrO_4 (400 Ci/g) was from New England Nuclear (Dorval, Quebec, Canada), [^{3}H]glycerol (11.5 Ci/mmol) and [^{3}H]inositol (15 Ci/mmol) were from American Radiolabeled Chemicals (St. Louis, MO), and [^{14}C]serotonin ([side chain 2-^{14}C]-hydroxytryptamine creatinine sulfate, 50 mCi/mmol) and BCS counting solution were from Amersham (Oakville, Ontario, Canada). Human α-thrombin (3,000 NIH units/mg), heparin (porcine intestinal mucosa, grade 1), amastatin, and HEPES were obtained from the Sigma Chemical Co. (St. Louis, MO). *Pasturella haemolytica* O-sialoglycoprotein endopeptidase (O-sialoglycoprotease) was from Cedarlane Laboratories, Ltd. (Hornby, Ontario, Canada). Albumin (fraction V) was from Boehringer Mannheim (Dorval, Quebec, Canada). Fibrinogen was prepared from human plasma by β-alanine precipitation [14], purified by DEAE-cellulose chromatography [15], followed by dialysis against 0.9% (w/v) saline solution [16], and finally delipidated by precipitation with 0.5 volumes of acetone at −5°C, after which the fibrinogen was resuspended in saline for use. Apyrase, prepared from potatoes by the method of Molnar and Lorand [17], was dissolved in 0.15 M NaCl and stored at −20°C. This preparation was added to 1 ml of platelet suspension in a volume that was sufficient to convert 0.25 μM ATP to AMP in 120 sec at 37°C. Silica gel H thin-layer chromatography plates (Si250) were from Canlab (Toronto, Ontario, Canada). Solvents for thin-layer chromatography were distilled before use. Precast Tris-glycine gels were from HELIXX Technologies, Inc. (Scarborough, Ontario, Canada). Polyclonal antibody GC-59593 was the generous gift of Dr. J.E.B. Fox (Cleveland Clinic Foundation, Cleveland, OH).

Platelet Preparation

Human platelets were isolated from whole blood collected from a forearm vein into acid citrate dextrose anticoagulant [18]. The platelets were washed once in Tyrode's solution, pH 7.35 (which contains Ca^{2+} [2 mM] and Mg^{2+} [1 mM], to which heparin (50 U/ml), albumin (0.35%), glucose (0.1%), and apyrase were added [16]. After centrifugation, the platelets were resuspended in a modified Tyrode's-albumin solution from which phosphate was omitted; 5 mM HEPES were added to the suspending medium to compensate for the loss of buffering capacity due to the removal of phosphate, and to keep the pH at 7.35. Isotopes for labeling were then added.

Studies of Changes in Inositol Labeling of Inositol Phosphates in SFLLRN- and Thrombin-Stimulated Human Platelets

Suspensions of human platelets were incubated for 1 hr at 37°C with [^{3}H]inositol (20 μCi/ml). In all experiments, platelets were treated with aspirin (0.55 mM) for

the last 15 min of the labeling incubation. Unincorporated isotope was removed by washing the platelets twice by centrifugation and resuspending them in Tyrode's solution (which contains phosphate, Ca^{2+}, and Mg^{2+}, and to which glucose, albumin, and apyrase were added). The platelets were finally resuspended at 5×10^8 platelets/ml in Tyrode's solution to which glucose, albumin, and apyrase were added.

Experiments were normally started 1 h after resuspension of the platelets, when labeling with [^{3}H]inositol of the inositol phospholipids had been found to be stable because it did not change over the following 2 hr (unpublished observations). However, since it was possible that the isotope had not completely equilibrated with all the platelet inositol phospholipids, paired control samples were done with all experimental samples. The platelets were stimulated with SFLLRN or thrombin (isotonic saline solution was used in control samples) while being stirred at 1,100 rpm in the cuvette of an aggregometer (Payton Associates, Scarborough, Ontario, Canada).

After the stirred platelets were stimulated with SFLLRN or thrombin, the reaction was stopped and the extraction of inositol phosphates initiated by the addition of trichloroacetic acid to 10% (w/v) and cooling on ice. The inositol phosphates were recovered and fractionated on ion-exchange columns (AG1-X8, Bio-Rad Laboratories Ltd., Mississauga, Ontario, Canada) [19].

Study of Specific Radioactivity of PIP_2 in SFLLRN- and Thrombin-Stimulated Human Platelets

For determination of the chemical amount and labeling of phosphatidylinositol 4,5-bisphosphate (PIP_2), the platelets were prelabeled as described in the previous section except that [^{3}H]glycerol (20 μCi/ml) was used and the platelets were finally resuspended at 10^9 platelets/ml. In all cases, platelets were aspirin-treated. The platelets were stimulated with SFLLRN or thrombin (isotonic saline solution was used in control samples) while being stirred at 1,100 rpm in the cuvette of an aggregometer (Payton Associates). Reactions were stopped by extraction of 1 ml of reaction mixture with 3.75 ml of chloroform/methanol (1:2), and the phospholipids were further extracted under acidic conditions, fractionated, and quantitated as previously described [20]. Values for the amount and labeling of PIP_2 were corrected for the efficiency of recovery, which had previously been determined by adding a known amount of PIP_2 labeled with [^{32}P]phosphate to unlabeled platelets and taking the mixture through the extraction and fractionation procedures. The recovery was 68%.

Studies of the Effect of Degradation of GPIb With O-sialoglycoprotease on Changes in Inositol Labeling of Inositol Phosphates in Thrombin-Stimulated Human Platelets

To examine the role of GPIb in stimulation of inositol phosphate formation by thrombin, GPIb was degraded using O-sialoglycoprotease [12]. After [^{3}H]inositol was added to the platelet suspension to begin labeling, the suspension was divided into two portions, and the O-sialoglycoprotease (10 μg/ml) was added to one portion and the incubation was continued for 1 hr. During the last 15 min of the incubation, platelets were treated with aspirin (0.55 mM). Incorporation of [^{3}H]inositol into platelets was the same in protease-treated and control samples. The rest of the preparation of the platelets was the same as described above. To test the effect of the protease treatment, samples of the final suspension were removed and solubilized for SDS-PAGE in Laemmli sample buffer [21] in the presence of a reducing agent [22], and the platelet proteins were separated on SDS-polyacrylamide gels containing an 8–16% gradient of acrylamide. GPIb in the samples was detected by incubating Western blots, which were obtained by the method of Towbin et al. [23] with the polyclonal antibody GP-59593. Antigen-antibody complexes were detected by the enhanced chemiluminescence method (ECL, Amersham, Oakville, Ontario, Canada). Degradation of GPIb was also assessed by examining ristocetin-induced agglutination of fixed platelets by plasma von Willebrand factor (vWF) [24].

Studies of Secretion of [^{14}C]serotonin and Loss of [^{51}Cr]chromium From SFLLRN-Stimulated Platelets

To examine the secretion of dense granule contents and the loss of chromium (as a cytoplasmic marker) from platelets stimulated with thrombin or SFLLRN, the platelets were prepared as described above, except that they were labeled with [^{14}C]serotonin (0.1 μCi/ml) and [^{51}Cr]Na_2CrO_4 (1 μCi/ml) for 15 min at the end of the 1-hr incubation in the first washing solution. In experiments to assess secretion of serotonin, imipramine (5 μM) was added to the final platelet suspension to block reuptake of secreted serotonin. Secretion of [^{14}C]serotonin or loss of chromium was determined by rapidly transferring the platelet sample from the aggregometer cuvette to a centrifuge tube 120 sec after the addition of the aggregating agent and then centrifuging at 12,000*g* for 1.5 min in an Eppendorf centrifuge (Brinkman, Rexdale, Ontario, Canada); radioactivity was then determined in a sample of the supernatant fluid, and the percent secretion or loss was calculated [25].

TABLE I. Increases in Inositol Phosphates Due to Thrombin or SFLLRN Stimulation, Compared With Control Samples*

		Agonist-stimulated increase in [^{3}H]inositol labeling (dpm/10^9 platelets)				
		Thrombin (units/ml)		SFLLRN (μM)		
Time (sec)		1	2.5	20	50	100
n		(8)	(2)	(8)	(7)	(5)
IP	10	344 ± 354	−1,047 ± 846	−19 ± 180	−129 ± 347	−236 ± 137
	60	932 ± 254[b]	1,273 ± 1092	964 ± 357[a]	1,237 ± 468	960 ± 226
IP_2	10	1,155 ± 179[c]	1,147 ± 183[b]	535 ± 89[c]	799 ± 85[c]	971 ± 83[c]
	60	2,667 ± 187[c]	2,972 ± 333[b]	1,088 ± 106[c]	1,109 ± 136[c]	1,158 ± 110[c]
IP_3	10	344 ± 33[c]	301 ± 58[b]	220 ± 21[c]	265 ± 33[c]	288 ± 25[c]
	60	541 ± 51[c]	406 ± 47[c]	103 ± 32[b]	162 ± 33[c]	185 ± 33[c]

*Data presented as means ± SEM are from up to eight experiments (n) with duplicate samples. Incorporation into control samples: for IP, 5,699 dpm/10^9 platelets; for IP_2, 1,761 dpm/10^9 platelets; and for IP_3, 576 dpm/10^9 platelets.
[a–c]$P < 0.05$, $P < 0.02$, $P < 0.01$, respectively, for the significance of the differences between agonist-stimulated and unstimulated samples.

Analysis of Data

For calculation of the amount of label in the PIP_2, values in individual experiments were standardized to a total [^{3}H]glycerol incorporation of 4×10^6 dpm/10^9 platelets, which were average results. For calculation of the amount of label in the inositol phosphates, values in individual experiments were standardized to a total incorporation of [^{3}H]inositol of 2×10^5 dpm/10^9 platelets. The data are presented as means ± standard errors of the means, and the significance of the data was assessed by paired t-tests.

RESULTS

Platelet Aggregation

Stimulation of aspirin-treated, washed human platelets with 20 μM SFLLRN or 1 U/ml of thrombin resulted in rapid shape change and full-scale aggregation. The time courses of the responses were identical within the limits of resolution of the aggregometer tracings, with aggregation reaching full scale between 24–30 sec in different experiments. When secretion of [^{14}C]serotonin in response to thrombin and SFLLRN was compared, it was found not to be significantly different (thrombin 87.6 ± 2.0%, SFLLRN 81.6 ± 2.3%, n = 6); no loss of ^{51}Cr, which would have been indicative of lysis, was detected.

Inositol Phosphate Changes

In initial studies, we observed significant differences in the increases in chemical amount and labeling with [^{3}H]glycerol of phosphatidic acid stimulated by thrombin and SFLLRN at 10 and 60 sec, which could be due to different extents of activation of phospholipase C by the two agonists (data not shown). Since the differences were relatively small and thus difficult to interpret, the formation of inositol phosphates was examined.

At 10 sec, thrombin at 1 U/ml strongly stimulated formation of inositol bisphosphate (IP_2) and IP_3 based on increased labeling with [^{3}H]inositol; inositol phosphate (IP) labeling was not significantly increased (Table I). The labeling of all three inositol phosphates was further increased at 60 sec. Stimulation of platelets with a higher concentration of thrombin (2.5 U/ml) did not cause significantly larger increases in IP_3 at either time, indicating that maximum stimulation was achieved with 1 U/ml of thrombin.

Stimulation of platelets with 20, 50, or 100 μM SFLLRN for 10 sec increased IP_2 and IP_3 significantly (Table I). Stimulation of platelets for 10 sec with 50 or 100 μM SFLLRN resulted in larger increases in IP_3 than with 20 μM SFLLRN, that were not significantly lower than those caused by thrombin. However, by 60 sec, the increases in labeling of IP_3 induced by all three concentrations of SFLLRN had decreased such that the labeling in the 60-sec sample was much less than that at 10 sec ($P < 0.01$). Between 10 and 60 sec, the increase in the SFLLRN-stimulated labeling of IP_2 was less than the increase stimulated by thrombin with all concentrations of SFLLRN. In contrast to the increases in IP_2 and IP_3 labeling, the increases in labeling of IP in SFLLRN-stimulated platelets at 60 sec were similar to the increases in IP labeling caused by thrombin (Table I).

PIP_2 Specific Radioactivity

The specific radioactivity of PIP_2 labeling with [^{3}H]glycerol was unchanged by thrombin or SFLLRN (Table II). These results indicate that changes in IP_3, IP_2, and IP measured by labeling with [^{3}H]inositol (which distributes similarly to [^{3}H]glycerol in the phospho-

TABLE II. Specific Radioactivity of PIP_2 in Washed, Aspirin-Treated Human Platelets Prelabeled With [^{3}H]glycerol and Stimulated by Thrombin (1 U/ml) or SFLLRN (20 μM)*

Time	Tyrode's solution	Thrombin	Tyrode's solution	SFLLRN
	[^{3}H]glycerol specific radioactivity (dpm/nmol)			
10 sec	11.6 ± 1.3	10.2 ± 1.2	11.1 ± 1.0	12.0 ± 1.3
Diff.	1.3 ± 0.9n.s.		0.8 ± 0.6n.s.	
60 sec	10.4 ± 1.0	10.4 ± 0.7	11.0 ± 1.1	11.2 ± 0.9
Diff.	0.01 ± 1.2n.s.		0.3 ± 0.2n.s.	

*Differences (Diff.) are the mean difference and SEMD between the SFLLRN- or thrombin-stimulated samples and the paired control to which only Tyrode's solution was added. Results are from three experiments with duplicate samples, and are presented as mean differences ± SEM; n.s. = not significant.

inositides; unpublished observations) are likely good measures of changes in actual chemical amount.

Effect of Amastatin on SFLLRN-Induced Inositol Trisphosphate Formation

To rule out the possibility that the decrease in IP_3 formation at 60 sec in SFLLRN-stimulated platelets was due to degradation of SFLLRN by ectopeptidases, platelets prelabeled with [^{3}H]inositol were stimulated with 20 μM SFLLRN for 60 sec in the absence and presence of 100 μM amastatin. Amastatin alone had no effect on inositol phosphate labeling in unstimulated platelets and did not affect the labeling of IP_3 at 10 sec. Amastatin also did not affect the decrease in IP_3 labeling observed at 60 sec after SFLLRN stimulation. In two experiments in which 1 U/ml of thrombin increased IP_3 labeling at 10 and 60 sec by 309 ± 101 and 502 ± 68 dpm/10^9 platelets, and 20 μM SFLLRN caused increases at 10 and 60 sec of 278 ± 75 and 149 ± 79 dpm/10^9 platelets, the increases caused by 20 μM SFLLRN in the presence of amastatin at 10 and 60 sec were 256 ± 97 and 181 ± 75 dpm/10^9 platelets.

Effect of Degradation of GPIb With O-sialoglycoprotease on Thrombin-Induced Inositol Phosphate Formation

Since GPIb has been reported to be a high-affinity receptor for thrombin [10], and since interaction of thrombin with GPIb might account for the more sustained formation of inositol phosphates in thrombin-stimulated platelets, the effect of degrading GPIb with O-sialoglycoprotease was examined. Based on Western blots of treated and untreated platelets using a polyclonal antibody for GPIb in two experiments, approximately 100% and 80% of the GPIb were degraded by protease treatment (Fig. 1). Based on a visual assessment, the ability of ristocetin and vWF to agglutinate fixed samples

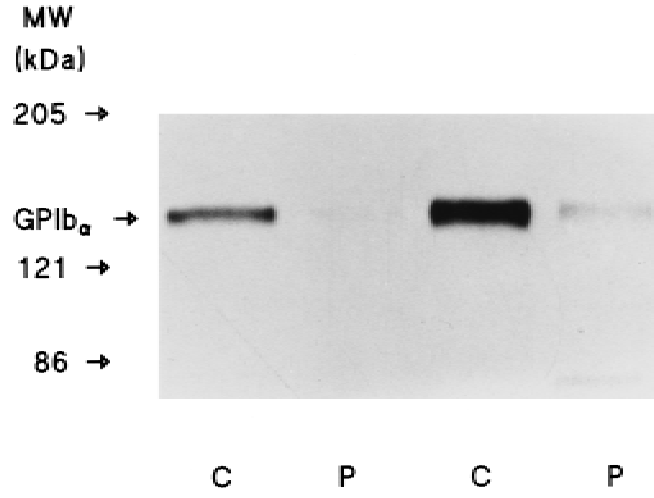

Fig. 1. Western blot of platelet proteins after treatment of intact platelets with O-sialoglycoprotease. Apparent molecular weight of GPIbα, based on comparison with MW standards, was calculated to be 148 kDa. In two experiments, the platelet suspension was divided into two portions. Half was treated with O-sialoglycoprotease (10 μg/ml for 1 hr at 37°C), and the rest was incubated without enzyme as the control preparation. Samples were prepared for Western blots as described in Materials and Methods; the remainder was used in the study reported in Table III. Control (C) and protease-treated (P) samples for both experiments are shown.

TABLE III. Comparison of Increases in Inositol Phosphates Due to Thrombin (1 U/ml) Stimulation of Platelets Treated With O-sialoglycoprotease or Untreated Platelets*

		Thrombin-stimulated increase in [^{3}H]inositol labeling (dpm/10^9 platelets)	
	Time	Untreated	Protease-treated
IP	10 sec	183 ± 58[a]	38 ± 109
	60 sec	745 ± 165[b]	782 ± 33[c]
IP_2	10 sec	697 ± 119[c]	487 ± 39[c]
	60 sec	1779 ± 108[c]	2019 ± 118[c]
IP_3	10 sec	369 ± 49[c]	328 ± 54[c]
	60 sec	596 ± 45[c]	683 ± 22[c]

*Data presented as means ± SEM are from two experiments with duplicate samples. None of the thrombin-stimulated increases in protease-treated samples were significantly different from those in untreated samples.
[a–c]$P < 0.05$, $P < 0.02$, $P < 0.01$, respectively, for the significance of the differences between agonist-stimulated samples and unstimulated samples.

of O-sialoglycoprotease-treated platelets was completely blocked, indicative of substantial degradation of the GPIb. However, as shown in Table III, the treatment had no effect on the thrombin-induced initial formation of inositol phosphates (10 sec) or on sustained formation at 60 sec. The protease treatment had no effect on SFLLRN-stimulation of inositol phosphate formation (data not shown).

TABLE IV. Comparison of Increases in Inositol Phosphates Due to Thrombin (1 U/ml) Stimulation of Platelets Incubated in the Presence and Absence of Calcium*

		Thrombin-stimulated increase in [^{3}H]inositol labeling (dpm/10^9 platelets)	
	Time	$+Ca^{2+}$	$-Ca^{2+}$
IP	10 sec	183 ± 441	317 ± 275
	60 sec	618 ± 979	1212 ± 865
IP_2	10 sec	1395 ± 358[a]	1354 ± 377[a]
	60 sec	3275 ± 570[c]	3177 ± 379[c]
IP_3	10 sec	269 ± 121[a]	220 ± 48[b]
	60 sec	443 ± 107[b]	448 ± 69[c]

*Data presented as means ± SEM are from two experiments with duplicate samples. None of the thrombin-stimulated increases in the absence of Ca^{2+} was significantly different from those in the presence of Ca^{2+}. The absence of Ca^{2+} was achieved by adding EGTA to a final concentration of 1 mM in a suspension of platelets prepared in Tyrode's-albumin solution that did not contain added Ca^{2+}.

[a–c]$P < 0.05$, $P < 0.02$, $P < 0.01$, respectively, for the significance of the differences between agonist-stimulated samples and unstimulated samples.

Effect of Influx of Extracellular Calcium on Thrombin-Induced Inositol Phosphate Formation

Others have suggested that platelet responses to thrombin and SFLLRN differ because thrombin but not SFLLRN can cause influx of extracellular Ca^{2+} [8]. To test this hypothesis, platelets were suspended in a Ca^{2+}-free Tyrode's medium, and 1 min before addition of thrombin, EGTA was added to a final concentration of 1 mM. The increases in inositol phosphates were the same in the absence and presence of Ca^{2+} (Table IV), indicating that sustained thrombin-induced inositol phosphate formation is not dependent upon influx of external Ca^{2+}. The absence of Ca^{2+} had no effect on the inositol phosphate formation induced by SFLLRN (data not shown).

DISCUSSION

The results from these experiments show that the changes in inositol phospholipid metabolism of human platelets induced by SFLLRN differ in some respects from those in response to thrombin; these differences may underlie differences observed by others in calcium mobilization. The results also indicate that interaction of thrombin with GPIb does not contribute to the observed differences.

The remarkable difference between thrombin stimulation and SFLLRN stimulation was seen at 60 sec when the rate of formation of IP_2 and IP_3 induced by SFLLRN, indicated by [^{3}H]inositol labeling, was decreased such that much less IP_3 was present at 60 sec than was found at 10 sec, irrespective of the concentration of SFLLRN used. The effect on IP_2 formation was not as dramatic; at 60 sec the labeling of IP_2 was slightly more than that at 10 sec with all concentrations of SFLLRN, and <50% of that stimulated by thrombin. However, the effect was consistent with transient activation of inositol phosphate formation by SFLLRN. These findings contrasted with the continuing increases in labeling of IP_3 and IP_2 in thrombin-stimulated platelets. The transient formation of IP_3 after stimulation of platelets with SFLLRN has a counterpart in the observations of Nieuwland et al. [7] of the reversible changes in pH, cytosolic Ca^{2+}, and pleckstrin phosphorylation which they found to be caused by SFLLRN (15 μM), compared to the irreversible changes due to thrombin (approximately 0.5 U/ml). Similar observations were made by Lasne et al. [26], who found that SFLLRN caused a weaker, more transient increase in cytosolic Ca^{2+} than thrombin, and by Kramer et al. [8], who suggested that the difference was due to failure of SFLLRN to cause Ca^{2+} influx. Our data, showing that preventing thrombin stimulation of Ca^{2+} influx by chelating extracellular Ca^{2+} with EGTA does not result in transient IP_3 formation, indicate that regulation of calcium mobilization is more likely at the level of IP_3 formation which regulates, rather than is regulated by, calcium levels.

Although the increases in IP_2 and IP_3 were less with SFLLRN than with thrombin, IP was not increased to a greater extent by SFLLRN than by thrombin. This observation indicates that the smaller increases in IP_2 and IP_3 caused by SFLLRN were not attributable to greater degradation of IP_2 and IP_3 to IP.

Several groups have demonstrated that terminating occupancy by thrombin of its platelet receptor, using hirudin, also stops inositol phospholipid hydrolysis and formation of phosphatidic acid, although aggregation and secretion are not inhibited [27–29]. To ensure that our observations were not due to degradation of SFLLRN, which would effectively terminate receptor occupancy, we used the aminopeptidase inhibitor amastatin to show that the decreased formation of IP_3 at 60 sec in SFLLRN-stimulated platelets was not caused by degradation of SFLLRN by ectopeptidase. Although degradation of a thrombin receptor-activating peptide (T-11) has been attributed to aminopeptidase M in plasma by Coller et al. [30], we found that amastatin did not affect the SFLLRN-induced changes in IP_3 at 60 sec in the suspensions of washed platelets that we used. Similar results were obtained by Lau et al. [9].

To examine the basis for the difference between thrombin- and SFLLRN-stimulated changes in inositol phospholipid metabolism, the effect of degrading GPIb, a potential receptor for thrombin but not SFLLRN, was examined. O-sialoglycoprotease has been reported to degrade platelet GPIb without affecting the thrombin receptor or GPIIb/IIIa [12]. Degradation of GPIb was confirmed by Western blots and assay of ristocetin-induced vWF agglutination of the platelets. Since treatment of platelets with O-sialoglycoprotease did not affect the sus-

tained formation of IP_3 stimulated by thrombin or its magnitude, it is unlikely that interaction of thrombin with GPIb could account for the ability of thrombin but not SFLLRN to cause the sustained stimulation of IP_3 formation.

Differences in inositol phospholipid metabolism have been identified in platelets stimulated with SFLLRN compared with thrombin. The transient nature of IP_3 formation caused by SFLLRN, such that less IP_3 was found at 60 sec than at 10 sec, contrasted with the stronger, sustained formation of IP_3 stimulated by 1 unit/ml thrombin, although the two agonists, at the concentrations used, caused similar, maximal extents of platelet aggregation and secretion. This observation extends that of Kramer et al. [8], who observed a difference in duration of Ca^{2+} mobilization and suggested differences in the signaling pathways stimulated by the two agonists. Our results, showing that SFLLRN-induced IP_3 formation is transient and that thrombin-induced inositol phosphate formation is not dependent on extracellular Ca^{2+}, indicate that transient IP_3 formation is not due to failure of SFLLRN to cause Ca^{2+} influx, but rather indicates that transient IP_3 formation can account for the transient responses, including Ca^{2+} mobilization, observed by Nieuwland et al. [7], Lasne et al. [26], and Kramer et al. [8]. The differences between the effects of SFLLRN and thrombin on phospholipase C activity indicate that other processes are involved in the stronger, sustained thrombin activation of phospholipase C, as has been suggested by others [26]. The study of the effect of degrading GPIb, a putative high-affinity receptor for thrombin [11], indicates that this receptor was unlikely to be involved in causing the stronger, sustained activation of inositol phospholipid metabolism found with thrombin in these aspirin-treated platelets. Finally, the finding of a transient formation of IP_3 in SFLLRN-stimulated platelets, but of full activation of platelet aggregation and secretion, indicates that the more sustained activation of phospholipase C caused by thrombin may have other roles, possibly related to later processes in which platelets participate.

ACKNOWLEDGMENTS

The technical assistance of Mr. S. Maric, Mr. A. Chahil, and Mr. D. Perry is gratefully acknowledged. We thank Dr. M. Rand (Department of Biochemistry, University of Toronto) for doing the Western blots. This research was supported by grants from the Heart and Stroke Foundation of Ontario (B-2447) and from the Medical Research Council of Canada (MT 1309). J.D.V. is a Career Investigator of the Heart and Stroke Foundation of Ontario.

REFERENCES

1. Coughlin SR: Thrombin receptor structure and function. Thromb Haemost 70:184, 1993.
2. Huang RS, Sorisky A, Church WR, Simons ER, Rittenhouse SE: Thrombin receptor-directed ligand accounts for activation by thrombin of platelet phospholipase C and accumulation of 3-phosphorylated phosphoinositides. J Biol Chem 266:18435, 1991.
3. Scarborough RM, Naughton MA, Teng W, Hung DT, Rose J, Vu T-KH, Wheaton VI, Turck CW, Coughlin SR: Tethered ligand agonist peptides. Structural requirements for thrombin receptor activation reveal mechanism of proteolytic unmasking of agonist function. J Biol Chem 267:13146, 1992.
4. Vouret-Craviari V, Van Obberghen-Schilling E, Rasmussen UB, Pavirani A, Lecocq J-P, Pouyssegur J: Synthetic α-thrombin receptor peptides activate G protein-coupled signaling pathways but are unable to induce mitogenesis. Mol Biol Cell 3:95, 1992.
5. Vassallo RR, Kieber-Emmons T, Cichowski K, Brass LF: Structure-function relationships in the activation of platelet thrombin receptors by receptor-derived peptides. J Biol Chem 267:6081, 1992.
6. Kinlough-Rathbone RL, Rand ML, Packham MA: Rabbit and rat platelets do not respond to thrombin receptor peptides that activate human platelets. Blood 82:103, 1993.
7. Nieuwland R, van Willigen G, Akkerman JWN: Different pathways for control of Na+/H+ exchange via activation of the thrombin receptor. Biochem J 297:47, 1994.
8. Kramer RM, Roberts EF, Hyslop PA, Utterback BG, Hui KY, Jakubowski JA: Differential activation of cytosolic phospholipase A2 (cPLA2) by thrombin and thrombin receptor agonist peptide in human platelets. Evidence for activation of cPLA2 independent of the mitogen-activated protein kinases ERK1/2. J Biol Chem 270:14816, 1995.
9. Lau L-F, Pumiglia K, Cote YP, Feinstein MB: Thrombin-receptor agonist peptides, in contrast to thrombin itself, are not full agonists for activation and signal transduction in human platelets in the absence of platelet-derived secondary mediators. Biochem J 303:391, 1994.
10. Brass LF: Issues in the development of thrombin receptor antagonists. Thromb Haemost 74:499, 1995.
11. Greco NJ, Jamieson GA: High and moderate affinity pathways for α-thrombin-induced platelet activation. Proc Soc Exp Biol Med 198: 792, 1991.
12. McGowan EB, Detwiler TC: Modified platelet responses to thrombin: Evidence for two types of receptors or coupling mechanisms. J Biol Chem 261:739, 1986.
13. Yeo E, Sutherland DR: Platelet GPIb is selectively cleaved by the Pasturella glycoprotease: Function and biochemical studies. Blood [Suppl] 82:612, 1993.
14. Straughn W III, Wagner RH: A simple method for preparing fibrinogen. Thromb Diath Haemorrh 16:198, 1967.
15. Lawrie JS, Ross J, Kemp GD: Purification of fibrinogen and the separation of its degradation products in the presence of calcium ions. Biochem Soc Trans 7:693, 1979.
16. Kinlough-Rathbone RL, Packham MA, Mustard JF: Platelet aggregation. In Harker LA, Zimmerman TS (eds): ''Methods in Hematology. Measurement of Platelet Function.'' Edinburgh: Churchill Livingstone, 1983, p 64
17. Molnar J, Lorand L: Studies on apyrases. Arch Biochem Biophys 93:353, 1961.
18. Aster RH, Jandl JH: Platelet sequestration in man. 1. Methods. J Clin Invest 43:843, 1964.
19. Vickers JD, Kinlough-Rathbone RL, Mustard JF: The decrease in phosphatidylinositol 4,5-bisphosphate in ADP-stimulated, washed rabbit platelets is not primarily due to phospholipase C activation. Biochem J 237:327, 1986.
20. Vickers JD: Extraction of polyphosphoinositides from platelets: Comparison of a two-step procedure with a common one-step extraction procedure. Anal Biochem 224:449, 1995.

21. Laemmli UK: Cleavage of structural protein during the assembly of the head of bacteriophage T4. Nature 227:680, 1970.
22. Packham MA, Livne AA, Ruben DH, Rand ML: Activation of phospholipase C and protein kinase C has little involvement in ADP-induced primary aggregation of human platelets: Effects of diacylglycerols, the diacylglycerol kinase inhibitor R59022, staurosporine and okadaic acid. Biochem J 290:849, 1993.
23. Towbin H, Staehelin T, Gordon J: Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. Proc Natl Acad Sci USA 76:4350, 1979.
24. Lu H, Soria C, Soria J, Deromeuf C, Perrot JY, Tenza D, Garcia I, Caen JP, Cramer EM: Reversible translocation of glycoprotein IB in plasmin-treated platelets—Consequences for platelet function. Eur J Clin Invest 23:785, 1993.
25. Greenberg J, Packham MA, Cazenave J-P, Reimers H-J, Mustard JF: Effects on platelet function of removal of platelet sialic acid by neuraminidase. Lab Invest 32:476, 1975.
26. Lasne D, Donato J, Falet H, Rendu F: Different abilities of thrombin receptor activating peptide and thrombin to induce platelet calcium rise and full release reaction. Thromb Haemost 74:1323, 1995.
27. Holmsen H, Dangelmaier CA, Holmsen H-K: Thrombin-induced platelet responses differ in requirement for receptor occupancy. J Biol Chem 256:9393, 1981.
28. Holmsen H, Dangelmaier CA, Rongved S: Tight coupling of thrombin-induced acid hydrolase secretion and phosphatidate synthesis to receptor occupancy in human platelets. Biochem J 222:157, 1984.
29. Huang EM, Detwiler TC: Thrombin-induced phosphoinositide hydrolysis in platelets. Receptor occupancy and desensitization. Biochem J 242:11, 1987.
30. Coller BS, Ward P, Ceruso M, Scudder LE, Springer K, Kutok J, Prestwich GD: Thrombin receptor activating peptides: Importance of the N-terminal serine and its ionization state as judged by pH dependence, nuclear magnetic resonance spectroscopy, and cleavage by aminopeptidase M. Biochemistry 31:11713, 1992.